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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 1/23/74

JAN 31 1974

1. Principal Investigator (give title and degrees):
Thomas L. Roszman, Ph.D., Assistant Professor

2. Institution & address:

College of Medicine
University of Kentucky
Lexington, Kentucky 40506

3. Department(s) where research will be done or collaboration provided:

Department of Cell Biology

4. Short title of study:
Effect of Substances Derived from Cigarette Smoke on the Immune Response

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The specific aims of this project are to investigate the effects of nicotine and the water soluble fraction from cigarette smoke on the immune response employing in vivo and in vitro models. More specifically, the proposed research is directed towards determining the effect of these substances on the: (a) in vitro and in vivo primary antibody response and (b) mitogen induced stimulation of murine lymphocytes.

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Source: https://www.industrydocuments.ucsf.edu/docs/slam0000

8. Brief statement of working hypothesis:

Cigarette smoking may alter or suppress the immune response. If the immune response is suppressed, increased incidences of infection and cancer are noted. The experiments proposed in this study are directed toward determining whether or not water soluble substances derived from cigarette smoke are immunosuppressive. Both in vitro as well as in vivo systems will be employed to assess the immunosuppressive potential of these substances.

9. Details of experimental design and procedures (append extra pages as necessary)

As appended.

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Source: https://www.industrydocuments.ucsf.edu/docs/sldm0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Department of Cell Biology is well equipped for the research to be undertaken in this proposal. Ample laboratory and office space is available. Available to the principal investigator are Zeiss spectrophotometers, refrigerated centrifuges, Spinco model L ultracentrifuges, liquid scintillation counters, fraction collectors, phase contrast microscopes, CO_2 incubators, cold rooms and electrophoresis equipment. Water baths, clinical centrifuges and a moderate supply of glassware are also available. The department has a central facility for washing glassware and media preparation. The Medical Center has a large central animal facility which is available.

11. Additional facilities required:

None.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Source: https://www.industrydocuments.ucsf.edu/docs/sldm0000

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Thomas L. Roszman	15%	2,730
Fringe Benefits		382
Technical		
Research Analyst (to be recruited)	100%	9,300
Laboratory Aide (to be recruited)	35%	1,462
Secretary (to be recruited)	50%	2,500
Fringe Benefits		1,060
	Sub-Total for A	17,434
B. Consumable supplies (by major categories)		
Glassware	800	
Chemicals	600	
Isotopes	500	
Animals (1000 mice at $1.40 each)	1,400	
Tissue culture supplies (this includes CO_2 tanks, serum, plasticware, antigen, mitogens, media and biochemicals)	2,000	
	Sub-Total for B	5,300
C. Other expenses (itemize)		
Media room services	400	
Travel (to attend FASEB meeting)	300	
Publication costs (reprints and page costs)	200	
Service contracts and maintenance	500	
Office supplies (duplicating and photography)	200	
Animal care (board costs of $0.022/day/mouse)	1,600	
	Sub-Total for C	3,200
	Running Total of A + B + C	25,934
D. Permanent equipment (itemize)		
	Sub-Total for D	--
	E	3,890
E. Indirect costs (15% of A+B+C)	Total request	29,824

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15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18,480	5,300	3,200	--	4,047	31,027
Year 3	19,600	5,300	3,200	--	4,215	32,315

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Source: https://www.industrydocuments.ucsf.edu/docs/sdmb0000

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Analysis of IgM Memory	Research Corporation 203-65-7H400-T3916	3,980	4/1/72 - open
The Effect of Cigarette Smoke on the In Vitro Secondary Antibody Response	University of Kentucky Tobacco and Health Research Institute 124-05-7H400-24036	25,154	7/1/73 - 6/30/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Effect of Cigarette Smoke on the Immune Response	University of Kentucky Tobacco and Health Research Institute	34,800	7/1/74 - 6/30/75
Effect of Cigarette Smoke on the Humoral Immune Response	Lung Association	13,938	7/1/74 - 6/30/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Thomas L. Roszman

Signature Thomas L. Roszman Date 1/24/74

Telephone 606-233-5913
Area Code Number Extension

Checks payable to

University of Kentucky Research Foundation

Mailing address for checks

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Univ. of Kentucky, Lexington, Ky. 40506

Responsible officer of institution

Typed Name Carl B. Delabar

Title Associate Director, U.K.R.F.

Signature [signature] Date 1-25-74

Telephone 606 233-5577
Area Code Number Extension

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Source: https://www.industrydocuments.ucsf.edu/docs/sdm0000

9. Experimental design and procedures

A. Background and Rationale:

It is widely accepted that cigarette smoking is injurious to health. In the Surgeon General's report (1) on health and cigarette smoking, a convincing volume of statistical data has been compiled indicating that a relationship between smoking and certain diseases does exist. However, there is as yet little experimental data which document and substantiate the statistical data. In particular, there are few reports concerned with the effect of cigarette smoking on the immune response. Recent advances in immunologic knowledge and technology now make it possible to employ reliable in vitro as well as in vivo models for studying the effect of exogenous factors on the immune response. Thus, the purpose of this proposal is to examine the effects of products derived from cigarette smoke on the immune response.

The broncopulmonary system is an important portal of entry for antigen into the body and has the capacity to mount a local immune response (2). Environmental factors such as air pollution and cigarette smoking may not only damage the function of this system but the local immune response as well. An increased susceptibility to respiratory infection has been linked to cigarette smoking and air pollution (3, 4). Inhalation of cigarette smoke, furthermore, can result in the absorption into the circulation of soluble substances derived from smoke. A case in point is nicotine which reaches a blood level of about 50 ng/ml of plasma after the smoking of one cigarette (5). If derivatives of cigarette smoke can be disseminated throughout the body and attain sufficient levels in lymphoid tissues suppression may result. Recent reports indicate that cigarette smoke in fact has an adverse affect on the immune response. For example, young adult cigarette smokers had a significantly lower level of antibody to influenza virus A_2 after either natural infection or vaccination as compared to a similar group of nonsmokers (6). Thomas, et al. (7) have studied the effect of cigarette smoke on the primary and secondary antibody response of mice. Mice were exposed for 26 weeks to the smoke of 30 cigarettes daily and theninoculated intratracheally with sheep red blood cells. The number of direct and indirect antibody-forming cells found in the lungs, lymph nodes and spleens of smoked and unsmoked animals was determined on various days after primary and secondary immunization. The results demonstrated that the immune response of all organs obtained from smoked mice was substantially lower than those obtained from unsmoked mice. In particular, the primary immune response of the lungs was completely abrogated. The results also indicated that soluble substances derived from cigarette smoke were entering the circulation and attaining sufficient levels in the spleens and lymph nodes to cause immunosuppression in these lymphoid tissues. These same workers have also noted that the responsiveness of lymphoid cells from smoked mice to phytohemagglutinin is markedly depressed after 35 weeks of exposure to cigarette smoke (8). The in vitro functional activity of lymphocytes and macrophages is also decreased by cigarette smoke and extracts from smoke. Izard and co-workers (9) have found that human peripheral blood lymphocytes exposed to the gas phase of cigarette smoke have a reduced capacity to undergo stimulation with phytohemagglutinin. Similar observations have been reported by Savel (10). Macrophage function is also impaired after in vitro exposure to cigarette smoke. Thus, Green and Carolin (11) have demonstrated that cigarette smoke depresses the in vitro bacteriocidal activity of rabbit alveolar macrophages. These investigators have further demonstrated that water

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soluble extracts from the gas phase of cigarette smoke can inhibit the glyceraldehyde phosphate dehydrogenase activity of these macrophages establishing a relationship between the loss of a vital enzyme activity and the depressed bacteriocidal capacity of these cells (12). Similar studies suggest that nicotine also interferes with the function of certain enzymes particularly dehydrogenases (13). Nicotine, furthermore, can depress the respiration rate as well as the adenosine triphosphatase activity of alveolar macrophages (14).

Work performed in this laboratory has been concerned with establishing the effect of nicotine and the water soluble fraction (WSF) from the whole smoke of the University of Kentucky reference cigarette 1R1 on the in vitro secondary antibody response to sheep red blood cells (15). Cell suspensions prepared from the spleens of rabbits previously immunized with sheep red blood cells were treated with various concentrations of either nicotine or WSF before or after antigenic stimulation. The resulting IgM and IgG response was dependent on the concentration of either nicotine or WSF which was added to the lymphoid cell cultures. A progressive suppression of the IgM and IgG antibody responses was observed after treatment of these cell cultures with concentrations of nicotine and WSF ranging from 1 to 100 ug/ml. These concentrations of nicotine and WSF are not cytotoxic to the spleen cells. In all cases the addition to the cultures of either nicotine or WSF ranging in concentration from 200 to 1000 ug/ml induced complete suppression of both the IgM and IgG responses. Suppression of the IgM and IgG responses, furthermore, was observed after exposing the cultures to either nicotine or WSF for a period of 2 hr before antigenic challenge indicating that these substances have a rapid and irreversible effect on immunocompetent cells possibly as a result of interfering with the early events involved in the induction of the antibody response and not with the actual synthesis of antibody. The results of these studies as well as those of others previously described suggest that cigarette smoke and products derived from cigarette smoke have immunosuppressive potential and that cigarette smoking may in fact cause suppression of the immune response with resulting increases in the incidence of microbial infections and neoplasias.

From the foregoing discussion, it is apparent that further studies are needed to more clearly define the effects of cigarette smoke on all aspects of the immune response. Experiments are required to determine the mechanism(s) of action of cigarette smoke induced immunosuppression. Attention also needs to be focused on the effect of cigarette smoke on the different types of cells involved in the immune response since it is now well-established that thymus dependent lymphocytes (T-cells), thymus independent lymphocytes (B-cells) and in certain cases macrophages collaborate with one another to initiate the humoral immune responses (16, 17, 18). There is preliminary evidence which indicates that certain T-cell and B-cell functions are sensitive to cigarette smoke or substances derived from cigarette smoke (9, 10, 19).

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B. Method of procedure:

The effect of nicotine and WSF on the in vitro primary antibody response. The first series of experiments will be concerned with determining the effect of nicotine and the water soluble fraction (WSF) from the whole smoke of the University of Kentucky reference cigarette 1R1 on the in vitro primary antibody response of spleen cells obtained from adult B $C3F_1$ (C57BL X C3A/ANF) mice to sheep red blood cells (SRBC). For each experiment the mice will be matched to age and sex.

Freshly prepared samples of nicotine and WSF will be obtained from the University of Kentucky Tobacco and Health Research Institute Core Service Smoke Chemistry Laboratory. Mouse spleen cell suspensions will be prepared and the primary in vitro antibody response to SRBC will be performed according to the method of Mishell and Dutton (20). In brief, the procedure for carrying out the in vitro response will be as follows. Spleen cell suspensions will be adjusted to $1X10^7$ nucleated cells/ml in Eagle's medium containing vitamins, glutamine, nonessential amino acids, antibiotics and 10% heat inactivated fetal calf serum (FCS). One ml volumes of the cell suspension will be placed in 35mm Petri plates and stimulated with $5x10^6$ SRBC. The cultures will be incubated at 37C on a rocker platform oscillating at 10 cycles/min. Nutrient cocktail and FCS will be added to the cultures daily (20). At the time of addition of the SRBC and at 24 hr intervals thereafter, aliquots of the spleen cells will be removed to determine the number of direct (IgM) plaque-forming cells (PFC) employing a modification of the Cunningham assay procedure (21). Cell viability will be determined initially and at 24 hr intervals employing the trypan blue dye exclusion procedure.

Initial experiments will be involved with determining the kinetics of the in vitro primary antibody response to SRBC. After completion of these experiments, the effect of various concentrations of nicotine and WSF on the in vitro response will be assessed as follows. To duplicate antigen stimulated spleen cell cultures will be added from between 0.1 ug to 1000 ug/ml of either sterile nicotine or WSF. The spleen cell cultures will be exposed to these substances for the duration of the culture period. The number of PFC, percentage of viable cells and the number of nucleated cells recovered will be determined from day o through day 6. The data from these experiments will provide information with regards to the effect of nicotine and WSF on the time course and magnitude of the antibody response as well as the effect on cell survival.

Since nicotine and perhaps WSF are rapidly metabolized and eliminated under in vivo conditions, experiments will be designed to determine what effect short-term exposure to these substances will have on the in vitro primary antibody response of spleen cell cultures. Moreover, these experiments will provide information with regards to the effect of these substances on the inductive and proliferative phases of the response. The experiments will be performed and data collected as previously described except that the spleen cell cultures will be exposed to the various concentrations of either nicotine or WSF for a period of 2 hr prior to antigenic stimulation or at the time of antigenic stimulation and removed 24 hr later. The nicotine and WSF will be removed by washing the spleen cells three times with Eagle's medium containing 1% FCS and the cells finally resuspended to their original volume in complete medium containing 10% FCS and the other additives. Experiments will also be carried out to determine

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the effect of nicotine and WSF on the proliferative phase of the in vitro primary response. Either nicotine or WSF will be added to the cultures at either 24 hr or 48 hr after antigenic stimulation. Twenty-four hr later the cultures will be washed to remove these substances and the cells resuspended in medium and assayed for PFC as previously described.

Assuming that nicotine as well as WSF do have an effect on the in vitro primary antibody response other than simply a cytotoxic effect further experiments are proposed to determine the effect of these substances on the spleenic macrophages and lymphocytes. It is now clearly established that the induction of the in vitro primary antibody response of mouse spleen cells to SRBC is dependent on the presence of macrophages (22, 23). Therefore, the mouse spleen cell suspensions will be separated into macrophage-rich and lymphocyte-rich populations according to the method of Mosier (22). Neither the macrophage-rich or lymphocyte-rich population alone in the presence of antigen will give rise to an in vitro primary response. However, recombination of these populations in the presence of antigen will yield a response. The basic premise of these experiments is to determine what effect nicotine and WSF have on these two separate and distinct cell populations. To perform these experiments either the macrophage-rich or lymphocyte-rich population will be exposed to either nicotine or WSF as previously described. Recombination experiments will be performed where, for example, treated macrophages will be added in sufficient numbers to a fixed number of untreated lymphoid cells in the presence of SRBC and the magnitude of the ensuing primary antibody response measured as previously described. Similar experiments will be performed employing treated lymphocytes which will be recombined with untreated macrophages. Suitable controls will be carried out to ensure that neither the macrophage-rich or lymphocyte-rich population alone will support a primary response but when combined will yield a response.

The effect of nicotine and WSF on the stimulation of murine lymphocytes by concanavalin A and lipopolysaccharide. If nicotine and WSF have an adverse affect on the lymphocyte-rich population as assessed from the results obtained with the in vitro primary response system, further experiments are proposed to determine whether or not these substances have a differential effect on T- and B-cells. It is well-known that lymphocytes can be stimulated with certain mitogens such as concanavalin A (con A) and bacterial lipopolysaccharides (LPS) to undergo DNA synthesis, blastogenesis and cell division. Moreover, LPS is specific for B-cells (24) where as con A has specificity for only T-cells (25). Cell suspensions will be prepared from the spleens of adult $BC3F_1$, mice. The cell concentration will be adjusted to 2×10^6 nucleated cells/ml in RPMI-1640 medium supplemented with 5% FCS, glutamine and antibiotics. One ml aliquots of the cell suspension will be pipetted into 12 x 75 mm plastic tubes and various concentrations of either nicotine or WSF ranging from 0.01 ug to 1000 ug added for various lengths of time as previously described. The cell suspensions will be stimulated with optimal concentrations of either con A or LPS. At 48 hr after the addition of the mitogens, one uc of H^3-thymidine will be added to each culture and 18 hr later the radioactivity incorporated into DNA will be measured (26). The percentage of viable cells will also be determined by the trypan blue method.

The effect of nicotine and WSF on the in vivo primary antibody response. It is recognized that the in vitro effects of products derived from cigarette smoke on the primary antibody response do not necessarily indicate

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that similar effects will invaribly occur in vivo. There are, for example, in vivo detoxifying mechanisms which are probably not operative in the in vitro system. A case in point is the very short in vivo half-life of plasma nicotine after the smoking of one cigarette (5) and it can be assumed that other substances derived from cigarette smoke may also be rapidly eliminated. Thus, it is important to determine the effect of nicotine and WSF on the in vivo primary antibody response to SRBC. Adult $BC3F_1$ mice matched to age and sex will be employed in these experiments. The acute toxicity of nicotine and WSF in mice will be determined initially to establish a range of suitable concentrations for use in the in vivo experiments. The LD50 will be determined by injecting from between 0.1 mg to 20mg/kg of either sterile nicotine or WSF in a constant volume into the lateral tail vein. Twenty mice will be used for each concentration of either nicotine or WSF. The concentration range to be employed for nicotine is predicated on the results of Stakandske (27) for NMRI strain white mice. No information is available on the WSF from the University of Kentucky reference cigarette 1R1 thus the dose range is emperical. Having established sublethal dose ranges of nicotine and WSF for $BC3F_1$ mice two basic types of experiments are planned. Groups of mice will be injected intravenously with a constant volume of various concentrations of nicotine and WSF either before or after immunization with SRBC as follows. Beginning 3 days before immunization and at 12 hr intervals groups of mice will be injected with various concentrations of either nicotine or WSF. Twelve hr after the final injection of these substances the mice will be immunized intravenously with $5x10^8$ SRBC. In the case of those mice receiving either nicotine or WSF after immunization with $5x10^8$ SRBC the following protocol will be used. Mice will be injected intravenously with various concentrations of either nicotine or WSF at either 24 hr or 48 hr after immunization. Control groups of mice will be similarly injected with diluent and SRBC. Five mice from each group will be sacrificed on days 4, 5, 6, 8, & 10 and the number of direct and indirect PFC found in the spleens determined (21). The data will be statistically analyzed to determine whether or not there are significant differences between the primary immune response of mice treated with nicotine and WSF and the response of untreated controls. These experiments will provide information on the question of whether or not nicotine and WSF interfere with inductive and proliferative phases of the in vivo primary antibody response.

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